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Transcriptional Regulation of the *phoPR* Operon in *Bacillus subtilis*

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When *Bacillus subtilis* is subjected to phosphate starvation, the Pho regulon is activated by the PhoP-PhoR two-component signal transduction system to elicit specific responses to this nutrient limitation. The response regulator, PhoP, and its cognate histidine sensor kinase, PhoR, are encoded by the *phoPR* operon that is transcribed as a 2.7-kb bicistronic mRNA. The *phoPR* operon is transcribed from two σ^A-dependent promoters, P_1 and P_2. Under conditions where the Pho regulon was not induced (i.e., phosphate-replete conditions or *phoR*-null mutant), a low level of *phoPR* transcription was detected only from promoter P_1. During phosphate starvation-induced transition from exponential to stationary phase, the expression of the *phoPR* operon was up-regulated in a phosphorylated PhoP (PhoP∼P)-dependent manner; in addition to P_1, the P_2 promoter becomes active. In vitro gel shift assays and DNase I footprinting experiments showed that both PhoP and PhoP∼P could bind to the control region of the *phoPR* operon. The data indicate that while low-level constitutive expression of *phoPR* is required under phosphate-replete conditions for signal perception and transduction, autoinduction is required to provide sufficient PhoP∼P to induce other members of the Pho regulon. The extent to which promoters P_1 and P_2 are activated appears to be influenced by the presence of other sigma factors, possibly the result of sigma factor competition. For example, *phoPR* is hyperinduced in a *sigB* mutant and, later in stationary phase, in *sigH*, *sigF*, and *sigE* mutants. The data point to a complex regulatory network in which other stress responses and post-exponential-phase processes influence the expression of *phoPR* and, thereby, the magnitude of the Pho regulon response.

Bacillus subtilis responds to phosphate starvation by inducing or repressing genes of the phosphate stimulon, comprising: (i) the phosphate starvation-specific Pho regulon, (ii) the σ^B-dependent general stress (σ^B-GS) regulon, and (iii) PhoP-PhoR/σ^B-independent phosphate starvation-inducible genes (2, 12, 15, 22). The σ^B-GS regulon has ~200 members (29, 34), while the Pho regulon presently has 31 members. Of the latter, five operons (*phoPR*, *phoB-ydhF*, *pstSCA-pstBA-pstBB*, *phoD-tatAD*, and *tuaABCDEFGH*) and seven monocistronic genes (*glpQ*, *phoA*, *tatCD*, *ykoL*, *yhaX*, *yhbH*, and *yttP*) are induced in response to phosphate starvation, and two operons (*tagAB* and *tagDEF*) are repressed. The alkaline phosphatases (APases) PhoA and PhoB (4, 16), the phosphodiesterase-APase PhoD (6), and the glycerophosphodiesterase GlpQ (2) generate new sources of inorganic phosphate (P_i) from organic sources, such as deacylated phospholipids, nucleic acids, and teichoic acid (3). PhoD is secreted by the twin-arginine transporter (Tat) pathway, some components of which are encoded by members of the Pho regulon (19). Pst, a high-affinity phosphate ABC transporter (35), facilitates the uptake of P_i at low P_i concentrations. Concomitant repression of the teichoic acid operons (*tagAB* and *tagDEF*) and induction of teichuronic acid operon (*tuaA* to *tuaH*) conserves phosphate by bringing about the controlled replacement of the phosphate-containing cell wall polymer teichoic acid with the non-phosphate-containing teichuronic acid (21, 24, 28). The functions of five putative Pho regulon genes (*ydhF*, *ykoL*, *yhaX*, *yhbH*, and *yttP*) are presently unknown (2, 32, 36).

During phosphate starvation, genes of the Pho regulon are regulated by the PhoP-PhoR two-component signal transduction system (39, 40). The PhoP response regulator is activated by its cognate sensor kinase, PhoR. Phosphorylated PhoP (PhoP∼P) induces the expression of the *phoPR* operon about threefold from a low constitutive level of expression (17, 30, 32) and is required for the induction or repression of other members of the Pho regulon (15).

Phosphate starvation also induces the σ^B-mediated general stress response, and the Pho and σ^B-GS regulons interact to modulate the levels to which each is activated. In the absence of the regulator of one of these regulons, the expression of the other regulon is activated to a higher level (2, 32). For maximal induction of the Pho regulon, the respiration signal transduction system, ResD-ResE, is required (15). If, despite these responses, phosphate starvation persists, a third response regulator, Spo0A, initiates sporulation and terminates the phosphate response by repressing *phoPR* transcription via AbrB and ResD-ResE (15, 17).

The induction or repression of Pho regulon genes is mediated by the binding of PhoP∼P to Pho box sequences: direct repeats of TT(A/T/C)ACA with a 5 ± 2-bp spacer (7). For efficient binding, four TT(A/T/C)ACA-like sequences with an 11-bp periodicity are required. In the case of genes induced by PhoP∼P, the PhoP-binding sites are located on the coding

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristic(s)	Source[a] or reference
B. subtilis		
168	*trpC2*	1
168-PR	*trpC2 phoR*Δ*Bal*I::Tc^r	30
ML6	*trpC2 sigB*Δ*Hin*dIII-*Eco*RV::Cm^r	18
168-PR-SB	*trpC2 phoR*Δ*Bal*I::Tc^r *sigB*Δ*Hin*dIII-*Eco*RV::Cm^r	ML6→168-PR
SWV119	*trpC2 pheA1 abrB*::Tc^r	44
168-AB	*trpC2 abrB*::Tc^r	SWV119→168
SWV215	*trpC2 pheA1 spo0A*::Km^r	44
168-0A	*trpC2 spo0A*::Km^r	SWV215→168
BHI	*trpC2 pheA1 spo0H*Δ*Hin*dIII-*Eco*RI::Cm^r	11
168-SH	*trpC2 spo0H*Δ*Hin*dIII-*Eco*RI::Cm^r	BHI→168
650	*trpC2 ilvB2 leuB16 spoIIAABC*::Cm^r	J. Errington
168-SF	*trpC2 spoIIAABC*::Cm^r	650→168
901	*trpC2 spoIIGA*::Km^r	43
168-SE	*trpC2 spoIIGA*::Km^r	901→168
Plasmids		
pBluescript II KS(+)	Phagemid cloning vector Ap^r (2.961 kb)	Stratagene
pNHP	pBluescript II KS(+) containing a 621-bp insert of *phoP* Ap^r (3.558 kb)	This study
pNHR	pBluescript II KS(+) containing a 1,530-bp insert of *phoR* Ap^r (4.467 kb)	This study
pPE	pBluescript II KS(+) containing a 451-bp insert of *phoP* Ap^r (3.4 kb)	This study
pET2816	164-bp *Xba*I-*Blp*I fragment of pET16b replaced with the 255-bp *Xba*I-*Blp*I fragment of pET28a(+) (Novagen) Ap^r (5.802 kb)	T. Msadek
pET-PhoP	pET2816 containing a 722-bp insert of *phoP* Ap^r (6.386 kb)	This study
pET-PhoR231	pET2816 containing a 1,049-bp insert of *phoR* Ap^r (6.713 kb)	This study

[a] Arrows indicate transformation from donor to recipient.

strand of the promoter region and on the noncoding strand of the promoter regions of PhoP~P-repressed genes (25).

In the work described here we have used a combination of Northern hybridization, primer extension analyses, gel shift assays, and DNase I footprinting to analyze the transcriptional regulation of the *phoPR* operon. We compared the binding of PhoP and PhoP~P to the promoter region of *phoPR* with that of two other putative members of the Pho regulon, namely *ykoL* and *yhaX*. In addition, the transcription of *phoPR* was studied in *phoR*, *sigB*, and *abrB* mutants as well as in a number of mutants deficient in various stages of sporulation. The data confirm the role of PhoP in the regulation of *phoPR* and identify two sigma A-like promoters (P_1 and P_2) with associated Pho boxes. Moreover, the extent to which P_1 and P_2 are activated appears to be influenced by the presence of other sigma factors, possibly due to competition between sigma factors for binding to core RNA polymerase.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. *B. subtilis* strains and plasmids are listed in Table 1. Strains were grown in Luria Bertani (LB) medium, low-phosphate medium (LPM; 0.42 mM P_i), or high-phosphate medium (5.0 mM P_i) (31). *E. coli* XL1-Blue (Stratagene Europe, Amsterdam, The Netherlands) was used as the host for plasmid constructions, and *E. coli* BL21(λD3) (Novagen, Madison, Wis.) was used for the production of PhoP-His_6 and PhoR231-His_6. When required, the concentrations of antibiotics were the following: for *E. coli*, 100 μg of ampicillin (Ap) per ml and 25 μg of kanamycin (Km) per ml; for *B. subtilis*, 6 μg of chloramphenicol per ml, 0.3 μg of erythromycin per ml, 25 μg of lincomycin per ml, 10 μg of Km per ml, and 12.5 μg of tetracycline per ml. Isopropyl-β-D-thiogalactopyranoside (IPTG) was used at 1 mM.

DNA manipulations and general methods. Plasmid and chromosomal DNA extraction, restriction endonuclease digestion, agarose gel electrophoresis, transformation of *E. coli* cells, PCR, and bioinformatical analyses were carried out as described previously (30, 33). Enzymes, molecular size markers, and deoxynucleotides were purchased from Roche Diagnostics, Ltd. (Lewes, United Kingdom), and from Amersham Pharmacia Biotech, Ltd. (Little Chalfont, United Kingdom).

Construction of plasmids. Plasmids pNHP and pNHR (Table 1) were constructed to prepare digoxigenin-labeled RNA probes for *phoP* and *phoR*, respectively. Primers NHP-FOR and NHP-REV (Table 2) were used for PCR amplification of a 621-bp fragment of *phoP*, and primers NHR-FOR and NHR-REV were used to amplify a 1,530-bp fragment of *phoR*. The PCR fragments were cloned into *Hin*dIII- and *Bam*HI-digested pBluescript II KS(+) and were transformed into *E. coli* XL1-Blue. The resulting plasmids, pNHP and pNHR, were confirmed by sequencing the inserted DNA and adjacent vector sequences.

Plasmid pPE (Table 1) was constructed to generate a sequencing ladder for primer extension analysis. A 451-bp fragment from the 5′ end of *phoP* was amplified by using primers PE-FOR and PE-REV (Table 2) and was cloned into *Bam*HI- and *Eco*RI-digested pBluescript II KS(+). The structure of pPE was confirmed by sequencing the inserted DNA and adjacent vector sequences.

For the production and purification of PhoP-His_6 and PhoR231-His_6 proteins a 734-bp fragment encoding PhoP and a 1,067-bp fragment encoding the cytoplasmic region of PhoR (from amino acid 231 to the C terminus) were amplified by using primer pairs PP-FOR/PP-REV and R231-FOR/R231-REV, respectively (Table 2). The *Bsp*HI-*Xho*I-digested PCR fragment containing *phoP* and the *Bsa*I-*Xho*I-digested PCR fragment containing *phoR*231 were ligated into *Nco*I-*Xho*I-digested pET2816 (Table 1), and the mixtures were used to transform *E. coli* XL1-Blue. The structures of resulting plasmids pET-PhoP and pET-PhoR231 (Table 1), respectively, were confirmed by sequencing the inserted DNA and adjacent vector sequences. The PhoP and PhoR231 proteins encoded by these plasmids contained His_6 tags at their C termini (i.e., PhoP-His_6 and PhoR231-His_6).

RNA extraction, Northern hybridization, and primer extension. Total RNA of the *B. subtilis* strains was extracted with phenol (27). Digoxigenin-labeled RNA probes specific for *phoP* and *phoR* were synthesized in vitro with T7 RNA polymerase, using *Hin*dIII-linearized pNHP and pNHR (Table 1), respectively, and the DIG Northern Starter kit (Roche Diagnostics GmbH, Mannheim, Germany).

For primer extension analysis, primers PEPH1 and PEPH2 (Table 2), complementary to the region encoding the N terminus of *phoP*, were 5′ end labeled with [γ-^{32}P]ATP (Amersham Pharmacia Biotech, Ltd.) by using T4 polynucleotide kinase (Promega). Total RNA (4 μg), ^{32}P-labeled primer, and SuperScript II RNase H$^-$ reverse transcriptase (Invitrogen Ltd., Paisley, United Kingdom) were used for the primer extension reaction. The primers used for reverse

TABLE 2. Primers[a]

Primer	Sequence (5′→3′)	Position (range)
NHP-FOR	CGCGAAGCTTGCACAGCATGAACAAGA	2977568 to 2977552
NHP-REV	CGCGGATCCTGCACATCAACAATTCTC	2976948 to 2976965
NHR-FOR	CGCGAAGCTTGGAAAGCAGAGGAACAC	2976737 to 2976721
NHR-REV	CGCGGATCCAATGCTTGACAATCGCTA	2975208 to 2975225
PE-FOR	CGCGGATCCACAGACTATGAAAGAGCG	2977844 to 2977827
PE-REV	CCGGAATTCAAGCATCACATCAAGCAC	2977394 to 2977411
PEPH1	GATTCTTCATCATCCACAACTA	2977524 to 2977545
PEPH2	GTAATGACATCATAGCCTGACC	2977470 to 2977491
PP-FOR	TCATCATGAACAAGAAAATTTTAGTTGTGGATGATGAAG	2977557 to 2977528
PP-REV	CTCCTCGAGTTCATTCATTTTTGGCTCCTCCAGTTTATACC	2976842 to 2976873
R231-FOR	GGTGGTCTCCCATGCAGCGGGATCGGCTGCTGAC	2976156 to 2976134
R231-REV	CTCCTCGAGGGCGGACTTTTCAGCGGCCCGTTTCAG	2975110 to 2975136
PhoP-FOR	GAGAGAAAGGCTTGCTTAATAC	2977766 to 2977745
PhoP-REV	AAATTTTCTTGTTCATGCTGTG	2977546 to 2977567
YhaX-FOR	TAACGATATTAGGGAGAATGGC	1055846 to 1055867
YhaX-REV	GAAGCAGCGCTCCATCTATATT	1056056 to 1056035
YkoL-FOR	TGAAATGCTGGAGACGTTTATG	1397308 to 1397329
YkoL-REV	TTTTCTAAAGCGGATTTCAATA	1397520 to 1397499

[a] Positions of the primers specific for *B. subtilis* 168 are with respect to the entire genome as noted in the SubtiList database (http://genolist.pasteur.fr/SubtiList) (20). The 5′ ends of primers included a 9- or a 10-bp linker with a *Bam*HI, *Bsa*I, *Bsp*HI, *Eco*RI, *Xho*I, or *Hin*dIII restriction site (underlined).

transcription were also used to prime dideoxy sequencing reactions from the corresponding *phoP* region of plasmid pPE (Table 1) as described previously (33).

Production and purification of PhoP-His$_6$ and PhoR231-His$_6$. Proteins PhoP-His$_6$ and PhoR231-His$_6$ were produced from *E. coli* BL21(λD3) carrying pET-PhoP or pET-PhoR231, respectively, as described previously (5).

Phosphorylation of PhoP by PhoR231. Purified PhoR231 (8 μM) was incubated in phosphorylation buffer (0.1 M Tris-HCl [pH 8], 0.2 M KCl, 4 mM $MgCl_2$, 8 mM $CaCl_2$, 0.5 mM dithiothreitol, 0.1 mM EDTA, 3.7% glycerol) in the presence of PhoP (24 μM) for 10 min at room temperature (RT), followed by incubation with 2.5 μM (40 μCi) [γ-^{32}P]ATP for 20 min at RT. The reaction was stopped by the addition of 1/5 volume of sodium dodecyl sulfate (SDS) blue loading buffer. The samples were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The dried gels were exposed to Hyperfilm ECL X-ray film (Amersham Pharmacia Biotech). The radioactive gel images were scanned with a PhosphorImager (Storm 860; Molecular Dynamics) and were quantified by using Quantity One software (version 4.3; Bio-Rad Laboratories, Hercules, Calif.).

Gel shift assay. For the preparation of DNA probes, fragments of the *phoPR*, *yhaX*, and *ykoL* promoter regions were amplified with primer pairs PhoP-FOR/PhoP-REV, YhaX-FOR/YhaX-REV, and YkoL-FOR/YkoL-REV. In each case the 5′ ends of the forward (FOR) primers were labeled with [γ-^{32}P]ATP by using T4 polynucleotide kinase (Promega). In the gel shift reactions, 4 μM PhoR231 and 0.1 μg of poly(dI-dC) (Sigma) per μl were incubated with 0, 24, 47, 71, and 95 μM PhoP in the presence or absence of 5 mM ATP for 15 min at RT (23). After addition of the DNA probe (1,000 cpm per μl) the mixture was incubated for a further 30 min. The samples were analyzed on a 6% native polyacrylamide gel by using Tris-glycine-EDTA buffer (38).

DNase I footprinting. The coding DNA strand was labeled as described for the gel shift assay. The noncoding strand was labeled with [^{32}P] by using reverse PCR primers. In the DNA binding reactions, a solution of 4 μM PhoR231, 0.05 μg of bovine serum albumin per μl, and 0.1 μg of poly(dI-dC) per μl was incubated with 0, 19, 38, 57, and 76 μM PhoP in the presence or absence of 5 mM ATP at RT for 15 min in binding buffer (20 mM sodium phosphate buffer [pH 8], 50 mM NaCl, 2 mM $MgCl_2$, 1 mM dithiothreitol, 10% glycerol). After addition of the DNA probe (500 cpm per μl) the mixture was incubated for a further 30 min. DNase I (0.1 U in 10 mM $MgCl_2$–5 mM $CaCl_2$) was added to the reaction mixture, and digestion was conducted for 1 min. The reactions were stopped with DNase I stop solution (0.4 M Na-acetate, 50 μg of calf thymus DNA [Sigma] per ml, and 2.5 mM EDTA). The samples were analyzed on a 6% polyacrylamide gel containing 7 M urea. A Maxam and Gilbert sequencing ladder (cleavage reactions at purine residues [A+G]) (38) was loaded on the same gel.

Protein assay. Protein concentration was determined by using the Bio-Rad protein assay kit.

SDS-PAGE. SDS-PAGE was carried out as described previously (38): a 16% separating gel in Tris-glycine buffer was used for the detection of PhoP-His$_6$ and PhoR231-His$_6$ proteins, with low-molecular-size-range prestained protein standards as size markers (Bio-Rad). The gels were stained with GelCode blue stain reagent (Pierce).

Enzyme and P$_i$ assays. APase production (32) and P$_i$ concentration (10) were determined as described previously.

RESULTS

Transcription of the *phoPR* operon. *lacZ* transcriptional reporter studies indicated that the *phoPR* operon exhibits constitutive low-level expression during exponential growth (i.e., P$_i$-replete conditions) and is induced in response to phosphate starvation (32): 4 h after the transition from exponential to stationary phase, the expression of *phoPR* was threefold higher than that in the exponential phase (17, 30). To confirm these data and to determine the size of *phoPR* transcript(s), Northern blot analyses were performed on RNA extracted from *B. subtilis* 168 at various times during growth and phosphate starvation-induced stationary phase (Fig. 1). The *phoPR* operon is located on a 2,742-bp region of the chromosome (Fig. 1B) between two terminators, T_{mdh} [bp 2977773 to 2977730 (20); $\Delta G = -19.8$ kcal/mol] and T_{phoPR} [bp 2975014 to 2974989 (20); $\Delta G = -13.8$ kcal/mol]. Using probes specific for *phoP* and *phoR*, a single prominent band was detected with an estimated size of 2.7 kb (Fig. 1C). Compared with data for exponential phase, the amounts of this transcript were approximately threefold higher during transition phase and in stationary phase. Minor bands at 2.1 and 1 kb, seen only with the *phoR* probe, are likely to represent degradation products that are missing regions homologous to the *phoP* probe. The putative processing sites at the 3′ ends of the 1-kb and the 2.1-kb products coincide with inverted repeat sequences located 132 bp (AAGAAGCAAA and TTCTTCGCTT) and 1,221 bp (TGCTCGATCT and ACGAGCTAAA) downstream of the translation start site of *phoR*.

Primer extension analysis identified two major transcriptional start sites for the *phoPR* operon (Fig. 2). The upstream transcriptional start site (TS_1) was mapped to a single nucle-

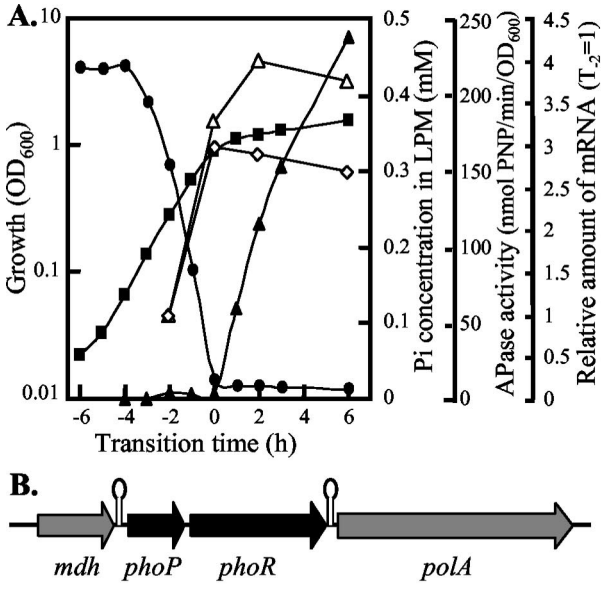

FIG. 1. Transcriptional activity of the *phoPR* operon. (A) *B. subtilis* 168 was grown in LPM. (■), optical density at 600 nm (OD_{600}); (▲), APase activities; (●), concentration of P_i in the medium. Also shown is the relative amount of mRNA as detected with probes specific for *phoP* (◇) and *phoR* (Δ). The relative amount of mRNA was calculated by using the intensities of the bands in the Northern hybridization experiments shown in panel C, normalizing the intensities with respect to the samples at T_{-2}. (B) Schematic representation of the *phoPR* region of *B. subtilis*. Filled thick arrows indicate structural genes while putative Rho-independent terminators are shown as stem-loop structures. (C) Total RNA, isolated 2 h before (T_{-2}) or at 0 (T_0), 2 (T_2), and 6 h (T_6) after entry into phosphate starvation-induced stationary growth phase, was used for Northern blot experiments with digoxigenin-labeled RNA probes specific for *phoP* (left-hand side) and *phoR* (right-hand side). The length (in nucleotide bases) of the molecular size marker is shown on the left-hand side of the images; the sizes (in nucleotide bases) of the three mRNA species of the *phoPR* operon are on the right-hand side. PNP, *p*-nitrophenyl.

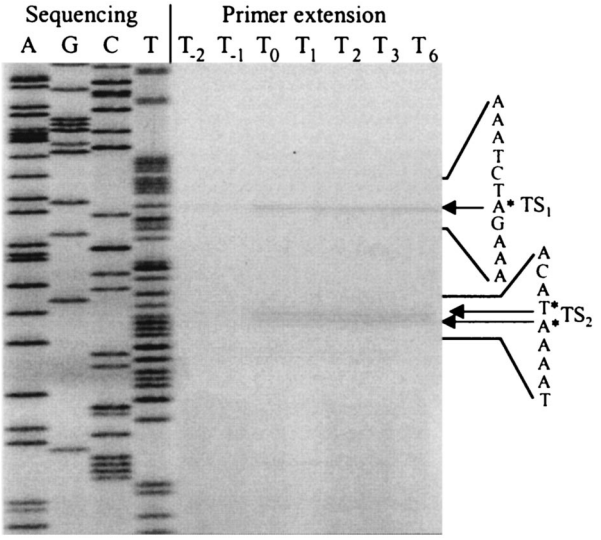

FIG. 2. Primer extension analysis of *phoPR* mRNA. Total RNA was isolated from *B. subtilis* 168 grown in LPM from T_{-2} to T_6 and was used as template for reverse transcriptase. The oligonucleotide primer PEPH1, used for reverse transcription, was also used to prime dideoxy sequencing reactions from the corresponding pPE plasmid (lanes A, G, C, and T). Positions of transcription start sites (TS_1 and TS_2) are labeled with asterisks and arrows.

otide (A) located 70 bp upstream of the *phoP* translational start site, while the second transcriptional start site (TS_2) mapped to two nucleotides (T and A) 49 and 48 bp upstream of the *phoP* translational start site. TS_1 was preceded by the sequence 5′ TTGTCG-N_{14}-CGCTAAAAT 3′ (P_1) (see Fig. 5), which is similar to the consensus sequence for a σ^A promoter (13). TS_2 was preceded by the sequence 5′ TAAAAT-N_{14}-TGTTAAGAT 3′ (P_2) (see Fig. 5), which has a −10 region similar to the consensus sequence for a σ^A promoter. However, the −35 region of P_2, which overlaps the −10 region of P_1, showed no homology to the σ^A consensus sequence. Because both promoters were induced in response to phosphate starvation (Fig. 2), the region upstream of the operon was analyzed for the presence of putative PhoP binding sites. Three TT(A/T/C)ACA-like sequences were located from 7 to 33 bp upstream of the −35 region of the P_1 promoter, while the P_2 promoter was associated with four TT(A/T/C)ACA-like sequences (see Fig. 5). In the latter case the location of the Pho box-like sequences from 28 bp upstream to 3 bp downstream of TS_2 is unusual for a PhoP-regulated promoter.

Binding of PhoP and PhoP~P to the region of the *phoPR* promoter. Gel shift assays and DNase I footprinting experiments were used to analyze the binding of PhoP to the promoter region upstream of *phoPR*. Because preparations of PhoP and PhoR were required for these experiments, PhoP-His_6 and PhoR231-His_6 variants of these proteins were overexpressed and purified from *E. coli* BL21(λD3) carrying plasmids pET-PhoP and pET-PhoR231, respectively (Table 1). Both protein preparations exhibited greater than 95% homogeneity, as determined by SDS-PAGE with the GelCode blue stain reagent (Fig. 3A). The functional activities of the purified proteins were determined with an in vitro phosphorylation assay. This confirmed that PhoR231-His_6 was autophosphorylated in the presence of [γ-^{32}P]ATP (Fig. 3B, lane 1) and was able to phosphorylate PhoP-His_6 (Fig. 3B, lanes 2 and 3).

Gel shift assays were performed to determine whether PhoP is able to bind to the promoter region of *phoPR* (Fig. 4A).

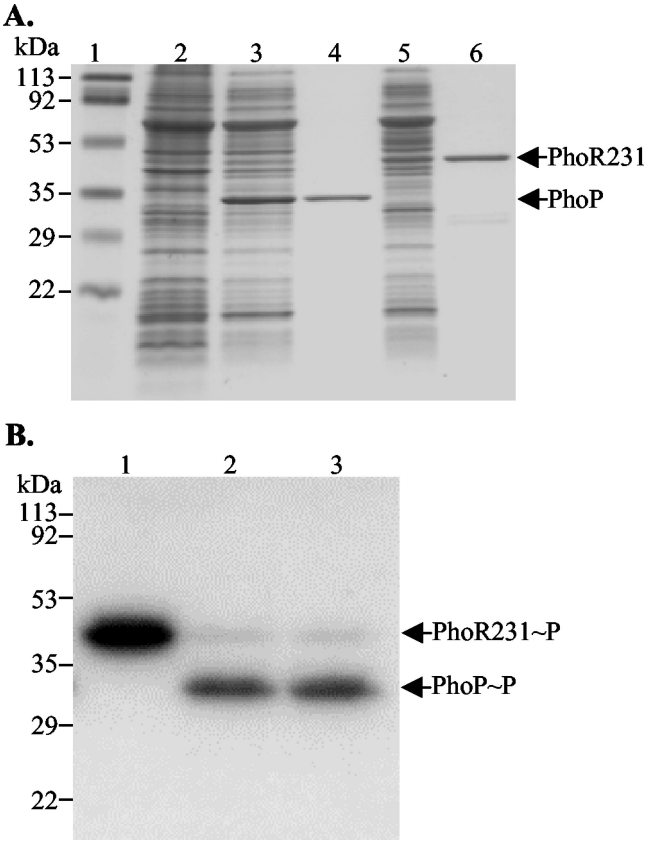

FIG. 3. Production and purification of PhoP-His_6 and PhoR231-His_6 and phosphorylation assay. (A) *E. coli* BL21(λD3) with pET-PhoP or pET-PhoR231 was grown in LB, and PhoP-His_6 and PhoR231-His_6 production was induced with IPTG. Purified proteins were analyzed by SDS-PAGE, and the gel was stained with GelCode blue stain reagent. An amount of 1 μg of protein was loaded in lanes 4 and 6, and 10 μg of protein was loaded in lanes 2, 3, and 5. Shown are prestained protein standards (lane 1), supernatant fraction of the whole-cell sonication lysate from the IPTG-induced *E. coli* BL21(λD3) carrying pET-PhoP (lane 3) or pET-PhoR231 (lane 5), and eluates of PhoP-His_6 (lane 4) and PhoR231-His_6 (lane 6) by 30 to 300 mM imidazole gradient. Supernatant fraction of the whole-cell sonication lysate from the noninduced *E. coli* BL21(λD3) carrying pET-PhoP (lane 2) was the negative control. (B) Phosphorylation of PhoP-His_6 by PhoR231-His_6 in the presence of ATP. PhoR231 (8 μM) was incubated with 40 μCi of [γ-^{32}P]ATP isotope in the absence (lane 1) or the presence (lanes 2 and 3) of 24 μM PhoP for 5 min (lane 1), 10 min (lane 2), and 20 min (lane 3) at RT. The samples were subjected to SDS-PAGE and, after being dried, the gel was exposed to an X-ray film.

PhoP and PhoP~P decreased the mobility of the ^{32}P-labeled *phoPR* promoter probe in a concentration-dependent manner. No significant differences were observed in the retardation of the probe when PhoP was phosphorylated, indicating that PhoP and PhoP~P bind to the *phoPR* promoter region in vitro with similar efficiencies. Compared to *ykoL* (Fig. 4B), relatively low amounts of the *phoP* probe were retarded by PhoP and PhoP~P, reflecting their relative in vitro levels of expression (~200 nmol *o*-nitrophenyl (ONP)/min/optical density unit for *ykoL-lacZ* and ~18 nmol ONP/min/optical density unit for *phoP-lacZ* at T_4) and induction ratios (~250-fold for *ykoL* and ~3-fold for *phoPR* at T_4) (32).

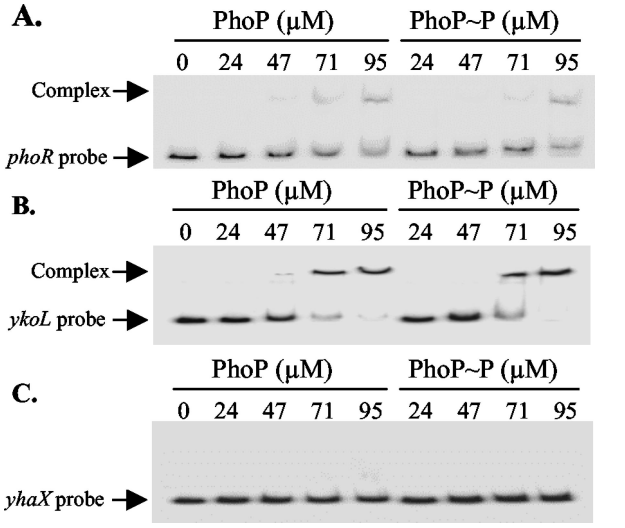

FIG. 4. Gel shift assays of the *phoPR*, *ykoL*, and *yhaX* promoter regions using PhoP and PhoP~P. PhoR231 (4 μM) was incubated with PhoP (0, 24, 47, 71, and 95 μM) in the absence (left-hand lanes) or presence (right-hand lanes) of ATP at RT for 15 min. ^{32}P-labeled *phoPR* (A), *ykoL* (B), and *yhaX* (C) promoter probes were added and, after binding (30 min), the samples were loaded onto a 6% native polyacrylamide gel to separate free DNA probe from DNA-protein complexes. After being dried the gel was exposed to X-ray film. The amounts of PhoP and PhoP~P added to the reaction mixtures are indicated above each lane.

DNase I footprinting was performed on both the coding and noncoding strands (Fig. 5) to locate the PhoP binding site on the *phoPR* promoter region. PhoP and PhoP~P protected the regions between −49 to −62 bp and −97 to −116 bp on the coding strand and between −53 to −70 bp and −115 to −145 bp on the noncoding strand. These regions correspond to the −10 sequence of promoter P_2 and the −35 sequence of promoter P_1. In addition, relatively more probe was bound by PhoP~P than by PhoP.

Binding of PhoP and PhoP~P to the promoters of *ykoL* and *yhaX*. The binding of PhoP and PhoP~P to the promoter region of *phoPR* was compared with that of two other putative members of the Pho regulon, namely *ykoL* and *yhaX* (32, 36). Gel shift assays and DNase I footprinting experiments were performed to determine if PhoP binds to the *ykoL* and *yhaX* promoter regions. PhoP and PhoP~P retarded the mobility of the *ykoL* promoter probe (Fig. 4B) in a concentration-dependent manner and to a greater extent than was observed for the *phoPR* promoter probe (Fig. 4A). DNase I footprinting of the *ykoL* promoter region showed that regions protected by PhoP and PhoP~P were located between +8 to −79 and −92 to −117 on the coding strand and between +30 to −65 and −92 to −111 on the noncoding strand (Fig. 6). Both PhoP and PhoP~P bound more efficiently to a region located at −35 to −63 on the coding strand and at −43 and −65 on the noncoding strand. In this region of the *ykoL* promoter, just upstream of the −35 sequence, the level of protection was significantly higher than was observed with the *phoPR* promoter (Fig. 5). The levels of protection afforded by the phosphorylated and nonphosphorylated forms of PhoP were similar. In contrast, the mobility of the *yhaX* probe was not influenced by PhoP and

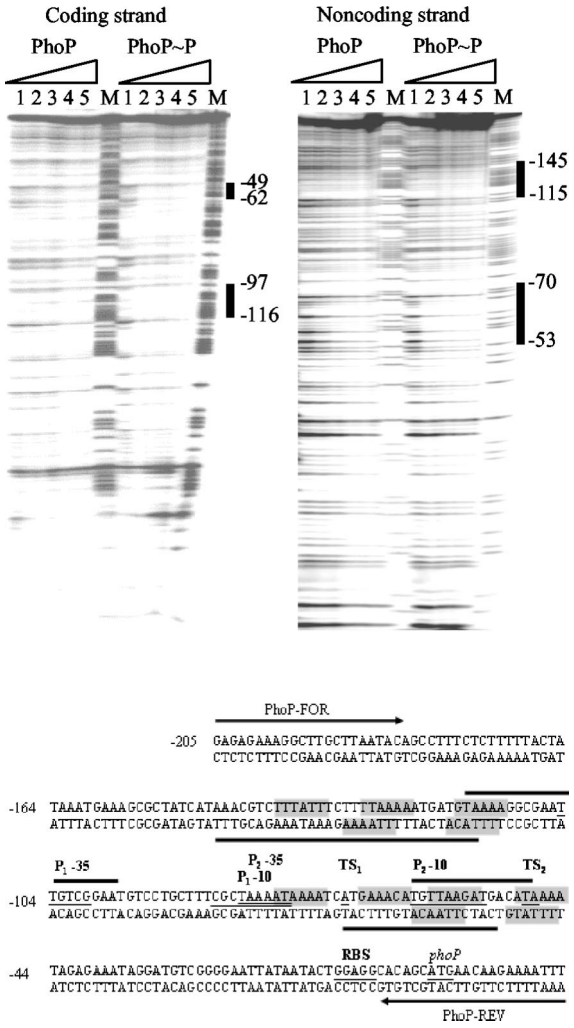

FIG. 5. DNase I footprinting assay of the *phoPR* promoter by using PhoP and PhoP~P. A PCR fragment corresponding to the *phoPR* promoter region (−205 to +16 relative to the translational start site of *phoP*) was used as the DNA probe. Coding strand footprinting ^{32}P-labeled PhoP-FOR primer and the noncoding strand footprinting ^{32}P-labeled PhoP-REV primer were used in the PCR to prepare the DNA probes. Increasing amounts (0, 19, 38, 57, and 76 μM; lanes 1 to 5) of PhoP were incubated with PhoR231 (4 μM) in the presence or absence of ATP and were mixed with the DNA probe. The thick black vertical lines show the regions where PhoP and PhoP~P bound. The numbers indicate the positions of the PhoP binding sites relative to the translational start site. M is the A+G Maxam and Gilbert sequencing reaction lane used as size markers. In the sequence of the *phoPR* promoter (lower part of the figure), the translational start site, ribosome binding site (RBS), transcriptional start sites (TS), and corresponding −35 and −10 sequences of the P_1 and P_2 promoters are underlined and labeled. Grey shading indicates direct repeats of TT(A/T/C)ACA for putative binding of the PhoP dimer (5 ± 2-bp spacer and maximum of two mismatches). The positions of PhoP-FOR and PhoP-REV primers are shown by thick arrows and are labeled.

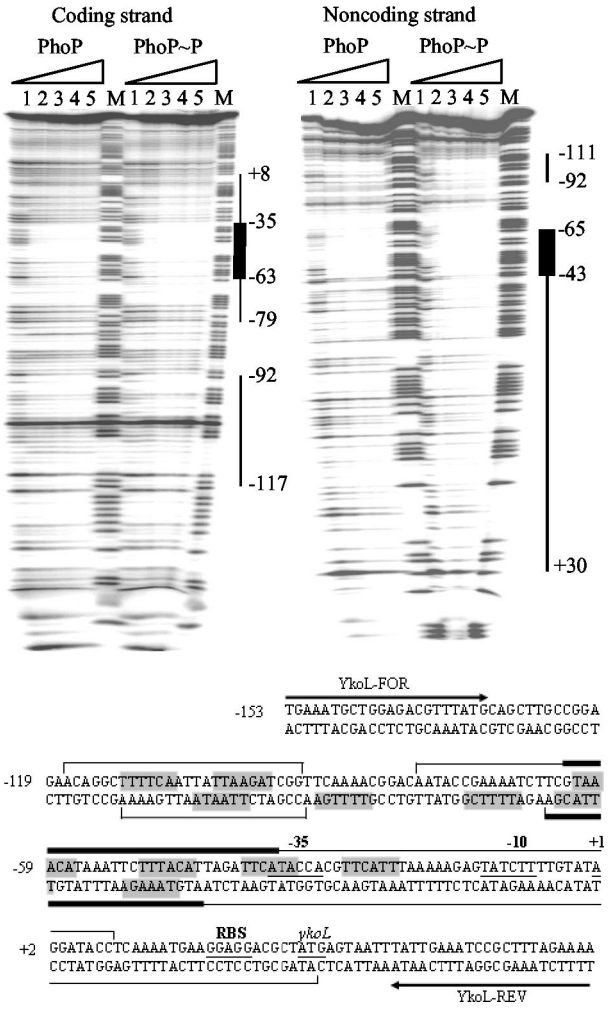

FIG. 6. DNase I footprinting assay of the *ykoL* promoter using PhoP and PhoP~P. A PCR fragment corresponding to the *ykoL* promoter region (−153 to +60 relative to the transcriptional start site of *ykoL*) was used as a DNA probe. Coding strand footprinting ^{32}P-labeled YkoL-FOR primer and the noncoding strand footprinting ^{32}P-labeled YkoL-REV primer were used in the PCR to prepare the DNA probes. Increasing amounts (0, 19, 38, 57, and 76 μM; lanes 1 to 5) of PhoP were incubated with PhoR231 (4 μM) in the presence or absence of ATP and were mixed with the DNA probe. The black vertical lines show the regions that were bound by PhoP and PhoP~P. The thick black lines show the regions where PhoP and PhoP~P bound with a higher affinity. The numbers indicate the positions of the PhoP binding sites relative to the transcriptional start site M is the A+G Maxam and Gilbert sequencing reaction lane used as size markers. In the sequence of the *ykoL* promoter the translational start site, RBS, transcriptional start site (+1), and corresponding −35 and −10 sequences of the promoter are underlined and labeled. Grey shading indicates direct repeats of TT(A/T/C)ACA for putative binding of PhoP dimer (5 ± 2-bp spacer and a maximum of two mismatches). The positions of YkoL-FOR and YkoL-REV primers are shown by thick arrows and are labeled.

PhoP~P in the gel shift assay (Fig. 4C), and these proteins showed no protective effect in the DNase I footprinting experiments (data not shown), indicating that there are no PhoP binding sites in the region of the *yhaX* promoter.

The effect of null mutations in the *phoR* and *sigB* genes on the transcription of the *phoPR* operon. Transcriptional studies with the *lacZ* reporter gene have shown that the induction of *phoPR* in response to phosphate starvation is enhanced threefold in a *sigB*-null mutant (32). In vitro transcription analyses were performed to determine the promoter(s) involved in this phenomenon. *B. subtilis* strains 168 (wild type), 168-PR (*phoR*), ML6 (*sigB*), and 168-PR-SB (*phoR*/*sigB*) were grown

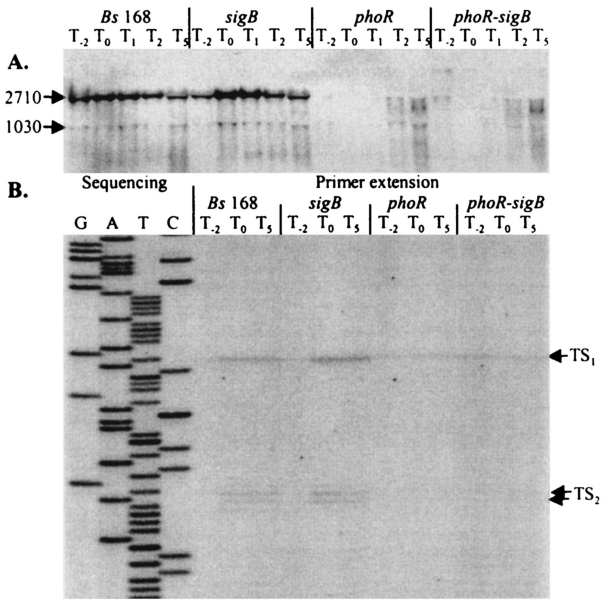

FIG. 7. The effect of *phoR*-null *sigB*-null mutations on the transcription of the *phoPR* operon. (A) For Northern hybridization analyses the *phoR*-specific mRNA probe and total RNA (4 μg), isolated from *B. subtilis* strains 168, 168-PR, ML6, and 168-PR-SB, were used. Bacteria were grown in LPM, and samples were taken 2 h before (T_{-2}) and at 0 (T_0), 1 (T_1), 2 (T_2), and 5 h (T_5) after entry into phosphate starvation-induced stationary growth. The sizes (in nucleotide bases) of the *phoPR* mRNA species are indicated on the left-hand side. (B) Primer extension analysis of *phoPR* mRNA. Total RNA, isolated from T_{-2}, T_0, and T_5, was used as template for reverse transcriptase. The oligonucleotide primer PEPH1 used for reverse transcription was also used to prime dideoxy sequencing reactions from the corresponding pPE plasmid (lanes G, A, T, and C). The positions of transcription start sites (TS_1 and TS_2) are labeled with arrows.

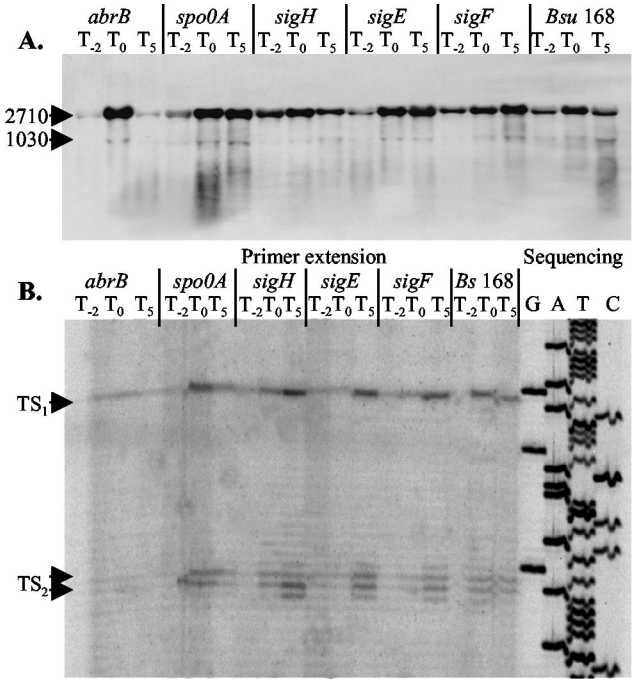

FIG. 8. Influence of mutations in *abrB*, *spo0A*, *sigH*, *sigF*, and *sigE* on the transcription of *phoPR*. (A) For Northern hybridization analyses the *phoR*-specific mRNA probe and total RNA (4 μg), isolated from *B. subtilis* strains 168, 168-AB, 168-0A, 168-SH, 168-SE, and 168-SF, were used. Bacteria were grown in LPM, and samples were taken 2 h before (T_{-2}) and at 0 (T_0) and 5 h (T_5) after entry into phosphate starvation-induced stationary growth. The sizes (in nucleotide bases) of the *phoPR* mRNA species are indicated on the left-hand side. (B) Primer extension analysis of *phoPR* mRNA. Total RNA, isolated from T_{-2}, T_0, and T_5, was used as template for reverse transcriptase. The oligonucleotide primer PEPH1 used for reverse transcription was also used to prime dideoxy sequencing reactions from the corresponding pPE plasmid (lanes G, A, T, and C). The positions of transcription start sites (TS_1 and TS_2) are labeled with arrows.

in LPM, and RNA was extracted throughout the growth cycle for Northern hybridization and primer extension analyses. Using a *phoR*-specific mRNA probe, Northern hybridization analysis showed that the level of induction of *phoPR* was twofold higher at T_0 and T_1 in the absence of SigB (Fig. 7A). Primer extension analysis (Fig. 7B) supported this result and showed that the P_1 and P_2 promoters were both up-regulated at T_0 and T_5. The data also confirmed that promoter P_1 was primarily responsible for the transcription observed during exponential growth (i.e., P_i-replete conditions) and in the absence of *PhoR* (i.e., *phoR*-null and *phoR*/*sigB*-null mutants). Evidence from the Northern hybridization experiments that the *phoPR* promoters were slightly induced at T_2 and T_5 in the *phoR*-null and *phoR*/*sigB*-null mutants (Fig. 7A) were not confirmed by the primer extension data (Fig. 7B). It is likely that the observed weak transcription was due to the presence in the *phoR*-null mutant of the tetracycline resistance gene (17).

The effect of mutations in *abrB*, *spo0A*, *sigH*, *sigF*, and *sigE* on the transcription of the *phoPR* operon. Transcription of *phoPR* is controlled by a regulatory network that includes, in addition to the PhoP and PhoR proteins, at least two other signal transduction systems, namely ResD-ResE and Spo0A (15). When activation of the Pho regulon fails to overcome the phosphate deficiency, phosphorylated Spo0A (Spo0A~P) initiates sporulation and indirectly represses *phoPR* transcription via AbrB and ResD-ResE (17). We therefore studied the effects on *phoPR* expression of mutations in genes encoding the transition state regulator AbrB and regulators controlling various stages of sporulation (Spo0A, SigH, SigF, and SigE). Northern hybridization analyses revealed that at the phosphate starvation-induced transition stage (T_0), *phoPR* transcription was induced about fourfold in the *abrB*-null mutant and two- to threefold in the wild type and sporulation-deficient mutants (Fig. 8A). In late stationary phase (T_5) the transcription of *phoPR* decreased slightly in the wild type and decreased markedly in the *abrB*-null mutant (Fig. 8A). However, the activities of both promoters P_1 and P_2 were induced in the sporulation-specific mutants (Fig. 8).

DISCUSSION

In *B. subtilis* the *phoPR* operon encodes the response regulator and histidine sensor kinase responsible for activating or repressing genes of the Pho regulon in response to phosphate starvation. This bicistronic operon encodes a single major transcript of 2.7 kb (Fig. 1C). Primer extension analysis revealed the presence of two transcriptional start sites, TS_1 and TS_2 (Fig. 2), which correspond to promoters P_1 and P_2. The −35

and −10 sequences of P_1 promoter and the −10 sequence of P_2 promoter were similar to the consensus sequence, TTGACA-N_{14}-TGNTATAAT, for σ^A promoters of *B. subtilis* (13). Our data indicate that the P_1 and P_2 promoters were active in *sigB* (Fig. 7B), *sigH*, *sigF*, and *sigE* (Fig. 8B) mutants, indicating that they are recognized by σ^A and not by these alternative sigma factors.

There is evidence that σ factors compete for a limiting pool of core RNA polymerase (9, 14, 26, 37), and data presented here and in previous work (32) are consistent with sigma factor competition affecting the expression of genes in the Pho regulon. For example, the Pho and σ^B-GS regulons are induced in response to phosphate starvation, and the cognate sigma factors σ^A and σ^B appear to compete for the core enzyme, because σ^A-dependent transcription of *phoPR* was enhanced twofold in a *sigB* mutant (Fig. 7A). Later in stationary phase σ^H, σ^F, and σ^E may compete with σ^A for the core enzyme, because the transcriptional activities of the two σ^A-dependent promoters of *phoPR* were significantly higher in the *spo0A*, *sigH*, *sigF*, and *sigE* mutants (stage 0, I, and II sporulation mutations) at T_5 than in the wild type (Fig. 8) and *sigB* mutants (Fig. 7A). However, we have not been able to provide direct evidence for sigma factor competition, and it is important to recognize that the inactivation of alternative sigma factors is likely to have pleiotropic effects on gene regulation that could also account for the data.

Transcription of the *phoPR* operon was higher in the *spo0A*-null mutant than in the wild type (Fig. 8A). This could be due to (i) direct repression by Spo0A∼P at putative 0A-boxes (TGNCGAA) (41) located at the −35 sequences of P_1 promoter (98 to 105 bp and 106 to 112 bp upstream of the translational start site of *phoP*), (ii) competition between Spo0A∼P- and PhoP∼P-activated regulons for $E\sigma^A$ holoenzyme, or (iii) indirect repression by Spo0A∼P via both AbrB and ResDE (17, 42).

The P_1 and P_2 promoters were induced at T_0 (Fig. 2), when the phosphate concentration in the medium decreased to below 0.1 mM (Fig. 1A). Gel shift assays and DNase I footprinting showed that both PhoP and PhoP∼P were able to bind in the region of *phoPR* promoters in vitro (Fig. 4A and 5). However, in vivo only PhoP∼P was able to induce the transcription of P_1 and P_2 under phosphate starvation, because constitutive low-level transcription from the P_1 promoter was observed in the *phoR*-null mutant (Fig. 7B).

The binding of PhoP and PhoP∼P to the *phoPR* promoter region was compared to that with the *ykoL* and *yhaX* promoter regions. Transcriptional reporter studies (32) showed that the σ^A-dependent promoter of *ykoL* (36) was induced 250-fold in response to phosphate starvation, while the σ^E-dependent promoter of *yhaX* (8) was induced 21-fold. The binding of PhoP and PhoP∼P to the *ykoL* promoter was very much more efficient than that to the weakly expressed *phoPR* promoters (Fig. 4A and B and 5 and 6). It was previously reported that *yhaX* was induced in response to phosphate starvation in a PhoP- and PhoR-dependent manner (32). However, the present studies failed to demonstrate in vitro binding of PhoP and PhoP∼P in the region of the *yhaX* promoter (Fig. 4C). This indicates either that *yhaX* is activated by PhoP indirectly via another regulatory pathway or that binding of PhoP∼P to the *yhaX* promoter region requires an additional factor(s).

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